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The Influence of AUG Codons in the Hepatitis C Virus 5′ Nontranslated Region on Translation

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The initiation of translation of hepatitis C virus (HCV) is cap-independent and mediated by an internal ribosome entry site (IRES) that is located in the 5′ nontranslated region (5′ NTR) of the viral genome. This 5′ NTR is relatively long and folds into a complex structure involving multiple hairpins and a pseudoknot. Within the sequence encompassing the IRES there are several AUG triplets. Some of these AUG codons are conserved between HCV genotypes and the related pestiviruses. In this study the 5 AUG codons (positions 13, 32, 85, 96, and 215) that are present in the 5′ NTR of the HCV H-strain have been mutagenized to determine their influence on HCV cap-independent translation. The effect of these mutations on the expression of a chloramphenicol acetyl transferase (CAT) gene was tested in vaccinia virus vTF7-3 infected Hep2 cells transfected with plasmids for the expression of a monocistronic HCV 5′ NTR-CAT mRNA. Mutating the AUG codons at positions 13, 32, and 215 does not have a significant effect on CAT expression, inactivating the AUG codons at either position 85 or position 96 severely impaired IRES function. To determine whether ribosomes scan the RNA to select the initiation site, AUG codons were inserted up- and downstream of the authentic HCV polyprotein translation initiation codon (position 342). Analysis of these mutants has revealed that the ribosome is unable to use an AUG codon that is placed either 7 nucleotides upstream or 8 nucleotides downstream of the inactivated AUG at position 342. These results indicate that when scanning is involved in the recognition of the translation initiating AUG, it is limited to a narrow region between nucleotides 335 and 350.

INTRODUCTION

Hepatitis C virus (HCV) is the causative agent of the majority of cases of posttransfusion hepatitis (Kuo *et al.,* 1989; Alter *et al.,* 1989). It is related to flavi- and pestiviruses and has been classified as a separate genus of the *Flaviviridae.* Members of this family are enveloped, positive stranded RNA viruses. Their genome encodes a single polyprotein that is co- and posttranslationally cleaved to yield the viral proteins (Grakoui *et al.,* 1993a,b; Hijikata *et al.,* 1993).

In contrast to the flaviviruses the 5′ nontranslated region (NTR) of both HCV and pestiviruses is relatively long (340–385 nucleotides) and contains multiple AUG codons. Sequence comparison of HCV and pestivirus 5′ NTR sequence has revealed several domains with a high percentage of sequence identity (Han *et al.,* 1991). In addition, it has been shown that the 5′ NTR of these viruses is folded into a complex, but overall very similar structure encompassing multiple stem-loop structures (Brown *et al.,* 1992) and an RNA pseudoknot (Le *et al.,* 1995; Wang *et al.,* 1995). For both HCV (Tsukiyama-Kohara *et al.,* 1992; Wang *et al.,* 1993) and the pestiviruses bovine viral diarrhea virus (Poole *et al.,* 1995) and classical swine fever virus (CSFV; Rijnbrand *et al.,* 1996) it has recently been established that the 5′ NTR sequence contains an internal ribosome entry site (IRES). From mutagenesis studies it was concluded that except for hairpin A (Fig. 1A) all other RNA stem-loop structures are essential for IRES functioning (Wang *et al.,* 1994, 1995; Rijnbrand *et al.,* 1995, 1996).

IRES mediated and cap-independent translation was first discovered for picornaviruses (for a recent review see Jackson and Kaminski, 1995). There is no similarity in sequence nor in RNA structure of the 5′ NTR between picornaviruses and HCV or pestiviruses. A common feature, however, is the presence of multiple AUG codons that are closely followed by in-frame stop codons (Fig. 1B). For poliovirus type 2 Lansing it has been shown that the seventh AUG codon in the 5′ NTR is important for efficient translation initiation (Pelletier *et al.,* 1988; Meerovitch *et al.,* 1991; Nicolson *et al.,* 1991). This AUG codon is part of a conserved pyrimidine/AUG motif (Yn-AUG) which is located at the 3′ end of all picornavirus IRES elements (Jang and Wimmer, 1990). It has been proposed that this conserved Yn-AUG picornavirus domain binds to 18S rRNA and thereby promotes binding of the ribosomal subunit to the picornavirus RNA (Kühn *et al.,* 1990; Nicolson *et al.,* 1991). For poliovirus it has been hypothesized that ribosomes enter the RNA at or near this picornavirus domain and scan the RNA until the polyprotein initiation

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codon is encountered to initiate translation (for a recent review see Jackson and Kaminski, 1995). In cardio- and aphthoviruses the ribosomes are predicted to bind to a similar motif; however, translation is initiated at the AUG codon of the Yn-AUG motif (Kaminski *et al.,* 1990; Davies and Kaufman, 1992; Pilipenko *et al.,* 1994).

As in picornaviruses the 5′ NTR of HCV and pestiviruses contains multiple AUG codons upstream of the AUG codon used for translation initiation (AUG^{orf}). In HCV the number of upstream AUGs varies between three and six, depending on the HCV genotype. The AUG codons at position 85 and 215 are conserved in nearly all HCV isolates sequenced to date and AUG codons are also present at corresponding positions in the 5′ NTR of pestiviruses.

This study is directed toward the role of AUG codons in the HCV-H 5′ NTR on translation. In addition, we have analyzed the potential of ribosomes to initiate translation at AUG codons that were engineered at different positions up- or downstream of the polyprotein initiation site.

MATERIALS AND METHODS

Enzymes, oligonucleotides, and bacterial strains

Recombinant DNA techniques were performed using standard procedures (Sambrook *et al.,* 1989). Restriction endonucleases, DNA and RNA polymerases, and T4 DNA ligase were obtained from Pharmacia, Promega, New England Biolabs, and Gibco BRL. Oligonucleotides were synthesized on a 391-DNA synthesizer (Applied Biosystems) or purchased from Eurogentec Inc. Plasmids were transformed into *Escherichia coli* DH5α or MC1061.

Construction of plasmids for the expression of RNAs containing WT or mutant HCV 5′ NTRs

Mutations were created in the expression plasmid pWT-CATT (Fig. 1B). pWT-CATT is a derivative of pWT-CAT (Rijnbrand *et al.,* 1995) in which a T7 transcription terminator sequence from pET3A (Rosenberg *et al.,* 1987) was cloned. Unless indicated otherwise, mutations were created using a PCR based strategy. The mutated sequences and their location are indicated in Figs. 3, 4, and 5. The nucleotide sequence of all mutants was verified.

AUG codons present in the 5′ NTR of HCV-H were mutated to non-AUG codons, resulting in pΔAUG^{13}-CATT, pΔAUG^{32}-CATT, pΔAUG^{85}-CATT, pΔAUG^{96}-CATT, and pΔAUG^{215}-CATT (Fig. 3). An adenosine, cytidine, guanosine, or thymidine residue was inserted at position 335 of the HCV-H 5′ NTR in pWT-CATT, resulting in p335+A(OF)-CATT, p335+C-CATT, p335+G-CATT, and p335+U-CATT (Fig. 4). p335+A(OF)-CATT was cut with either *Kpn*I or its isoschizomer Asp718, resulting in either 3′ or 5′ protruding ends. The protruding ends were subsequently polished by T4 DNA polymerase prior to ligation, resulting in p335+A(IF)-CATT and p335+A(NF)-CATT (Fig. 4).

A CAT module containing an initiation codon at the 5′ end was created by introducing the sequence AUGAG downstream of the *Kpn*I site. This *Kpn*AUG-CAT module was subsequently cloned downstream of a mutant 5′ NTR module in which the authentic initiation site was mutated to an ACG codon (pΔAUG^{orf}-CATT), resulting in pΔAUG^{orf}+14AUG-CATT. This plasmid was cut with either *Kpn*I or Asp718 and protruding ends were polished prior to ligation, as described above. The resulting plasmids were named pΔAUG^{orf}+10AUG-CATT and pΔAUG^{orf}+18AUG-CATT.

Construction of plasmids expressing RNAs without a functional HCV IRES

*Stu*I–*Hin*dIII fragments from pWT-CATT, p335+A(IF)-CATT, pΔAUG^{orf}+10AUG-CATT, pΔAUG^{orf}+18AUG-CATT, and pΔAUG^{orf}-CATT were cloned into *Hin*cII–*Hin*dIII-cut pBluescript(SK). The resulting plasmids pΔIRES-WT-CATT, pΔIRES-335+A(IF)-CATT, pΔIRES-ΔAUG^{orf}+10AUG-CATT, pΔIRES-ΔAUG^{orf}+18AUG-CATT, and pΔIRES-ΔAUG^{orf}-CATT contain only nucleotides 279 to 341 of the HCV 5′ NTR and 8 nucleotides of the coding region of the HCV-H strain fused to a CAT reporter gene.

Tissue culture and transient expression assay

Hep2 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 8% fetal calf serum. Near confluent monolayers of Hep2 cells, grown in 4-cm^2 dishes, were infected at a m.o.i. of 10 with the vaccinia virus recombinant vTF7-3 (Fuerst *et al.,* 1986), expressing T7 RNA polymerase. After an incubation at room temperature for 45 min the inoculum was removed and cells were transfected with 200 μl of DMEM containing 2.5 μg of CsCl-gradient purified plasmid DNA and 7.5 μl of lipofectin (Gibco BRL). Six hours post-transfection cells were lysed and analyzed for CAT expression by using the CAT enzyme linked immunosorbent assay kit (Boehringer Mannheim). CAT values were adjusted for differences in protein concentration of the lysates. Protein was determined using a Lowry based protein quantification assay (Sigma). The CAT expression levels presented in this study are the average of three transfections, except for the data presented in Fig. 3B for which $n = 6$. Error bars represent the standard error of the means.

In vivo labeling and immunoprecipitations

Transfections and infections were performed in 9.6-cm^2 wells similar as described above. Five hours after transfections the cells were starved for 30 min in DMEM without methionine and cysteine. After this period the cells were incubated for 1 hr in 500 μl DMEM without methionine and cysteine containing 100 μCi expre^{35}s^{35}s[^{35}S]Protein labeling

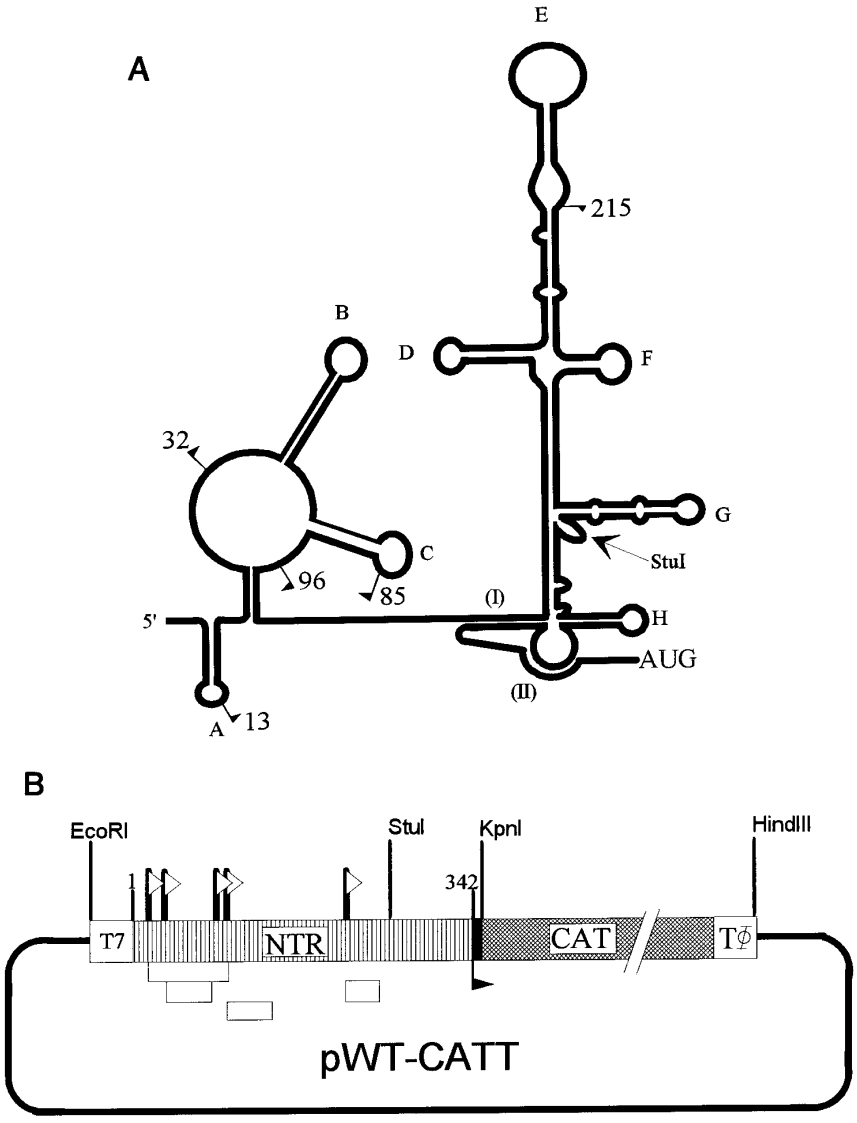

FIG. 1. (A) Secondary structure model of the HCV 5′ NTR. RNA stem-loop structures are labeled A to H. The two stems of the RNA pseudoknot are indicated by I and II. Flags and numbers refer to the position of the AUG codons in the HCV 5′ NTR. (B) Diagram of the expression plasmid pWT-CATT that was used for the expression of HCV 5′ NTR-CATT RNA. T7, T7 RNA promoter; TΦ, T7 transcription terminator; hatched box, HCV 5′ NTR; cross-hatched box, CAT gene; solid box, HCV capsid protein coding region. *Eco*RI, *Stu*I, *Kpn*I, and *Hin*dIII, restriction enzyme sites. The open reading frames in the 5′ NTR and their AUG codons are indicated by white boxes and flags, respectively. The polyprotein initiation site is indicated by a black flag.

mix (1175 Ci/mmol, NEN). Cells were lysed in TESV (300 μl/dish; 20 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, 100 m*M* NaCl) containing 1% Triton X-100 and 2 m*M* phenylmethylsulfonyl fluoride. Expression of CAT protein was analyzed by radioimmunoprecipitation (Vennema *et al.,* 1990) using saturating amounts of CAT antibody (5prime → 3prime Inc.). Immunoprecipitations were analyzed by SDS–PAGE.

RESULTS

Mutational analysis of the upstream AUG codons in the HCV-H 5′ NTR

The 5′ NTR of the HCV-H strain contains five AUG codons (Fig. 1), two of which (AUG^{85} and AUG^{215}) are highly conserved in HCV and pestiviruses. Mutant ΔABC-CATT (Rijnbrand *et al.,* 1995) which lacks AUG^{13}, AUG^{32}, AUG^{85}, and AUG^{96} is able to direct translation in vTF7-3 infected Hep2 cells (Fig. 2). This is not due to IRES mediated translation as deletion of either hairpin B or C is sufficient to abolish IRES activity in this system (Fig. 2). The translation of ΔABC-CATT is, however, dependent on the vTF7-3 system as no CAT was produced with the same mutants in either reticulocyte lysates or BT7-H cells (Rijnbrand *et al.,* 1995). A small fraction of the T7 RNA polymerase generated RNAs is capped in vTF7-3 infected cells (Fuerst and Moss, 1989) and this most likely results in the translation of ΔABC-CATT RNA. This implies that ribosomes are able to scan for the

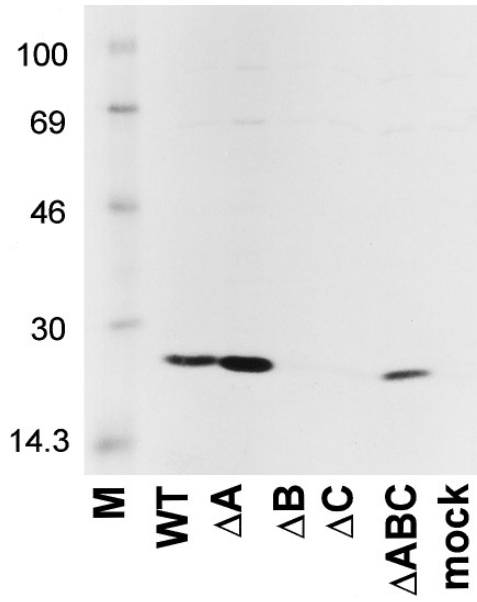

FIG. 2. Immunoprecipitation of CAT protein from radiolabeled, vaccinia vTF7-3 infected Hep2 cells transfected with the expression plasmids pWT-CATT, pΔA-CATT, pΔB-CATT, pΔC-CATT, and pΔABC-CATT, respectively.

polyprotein initiation site from the 5′ end of an RNA in which hairpins A, B, and C are deleted. This implies that if ribosomes would bind to the WT RNA at or just downstream of hairpin C, the ribosome with the cooperation of translation initiation factors can unwind the secondary structures present between hairpin C and the polyprotein initiation site.

For poliovirus it has been demonstrated that an AUG codon located upstream of the polyprotein initiation site is an important element of the IRES. To determine whether upstream AUG codons also play a role in HCV translation initiation we converted them to non-AUG codons (Fig. 3A). Based on the model for the folding of the HCV 5′ NTR (Brown *et al.,* 1992) the introduced mutations are not expected to alter the secondary structure of the RNA. For the mutants pΔAUG^{13}-CATT and pΔAUG^{32}-CATT (see legend of Fig. 3A for nomenclature) the sequence was changed to the sequence of the HCV 1b genotype that does not contain these two AUG codons (Smith *et al.,* 1995). For the other AUG codons mutations were introduced by altering one nucleotide in the codon either U → C (pΔAUG^{85}-CATT and pΔAUG^{215}-CATT) or A → G (pΔAUG^{96}-CATT). The translation of the mutants was analyzed in transfected and vTF7-3 infected Hep2 cells. The result of this experiment is depicted in Fig. 3B. Mutations in AUG^{13}, AUG^{32}, or AUG^{215} did not significantly change CAT expression as compared to pWT-CATT. Interestingly, a dramatic reduction in CAT expression was observed in lysates of cells transfected with either pΔAUG^{85}-CATT or pΔAUG^{96}-CATT, suggesting that the integrity of these AUG codons located in or close to hairpin C is essential for HCV-H IRES driven translation. The CAT expression levels do not result from differences in CAT mRNA stability, since no significant differences in CAT RNA abundance in the transfected cells could be detected by Northern blot analysis (data not shown).

Does scanning for the polyprotein initiation site occur from hairpin C?

The result of the mutational analysis of the upstream AUG codons combined with our observation that ΔABC-CATT is able to direct translation may imply that ribosomes bind at or near hairpin C and then start scanning the RNA. To address this possibility, an AUG codon seven nucleotides upstream of AUG^{orf} was created by introducing an adenosine residue at position 335

A

CONSTRUCT	ORIGINAL SEQUENCE	MUTATED SEQUENCE
ΔAUG^{13}	CUGAUGG	CgauUGG
ΔAUG^{32}	ACCAUGA	ACCAUag
ΔAUG^{85}	GCCAUGG	GCCAcGG
ΔAUG^{96}	AGUAUGA	AGUgUGA
ΔAUG^{215}	UCAAUGC	UCAcUGC

B

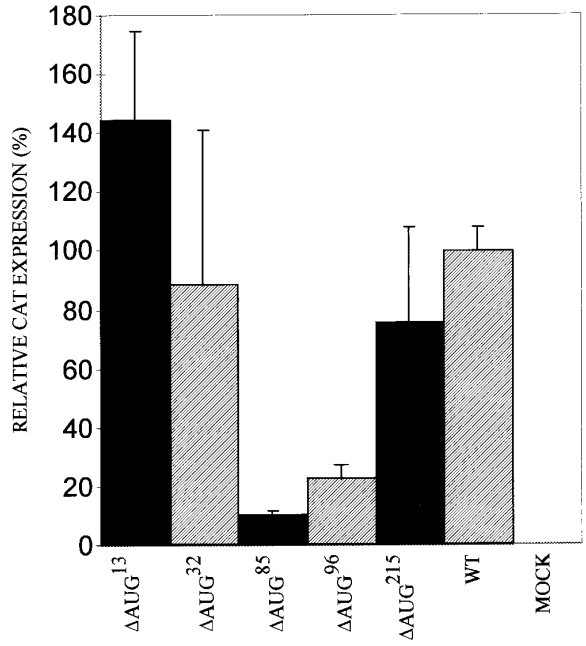

FIG. 3. The effects of inactivating the AUG codons in the HCV 5′ NTR. (A) The position of the AUG codon in the HCV 5′ NTR is given in the left column, followed by the nucleotide sequence surrounding that AUG codon. The mutations introduced to inactivate these AUG codons are shown in lowercase letters in the right-hand column. The position of the adenosine residue of the AUG nucleotide sequence has been used to mark its position in the HCV 5′ NTR. (B) Relative amount of CAT protein produced in vaccinia vTF7-3 infected Hep2 cells transfected with plasmids for the expression of monocistronic RNAs containing an inactivated HCV 5′ NTR AUG codon. CAT expression obtained in pWT-CATT transfected cells was set at 100%.

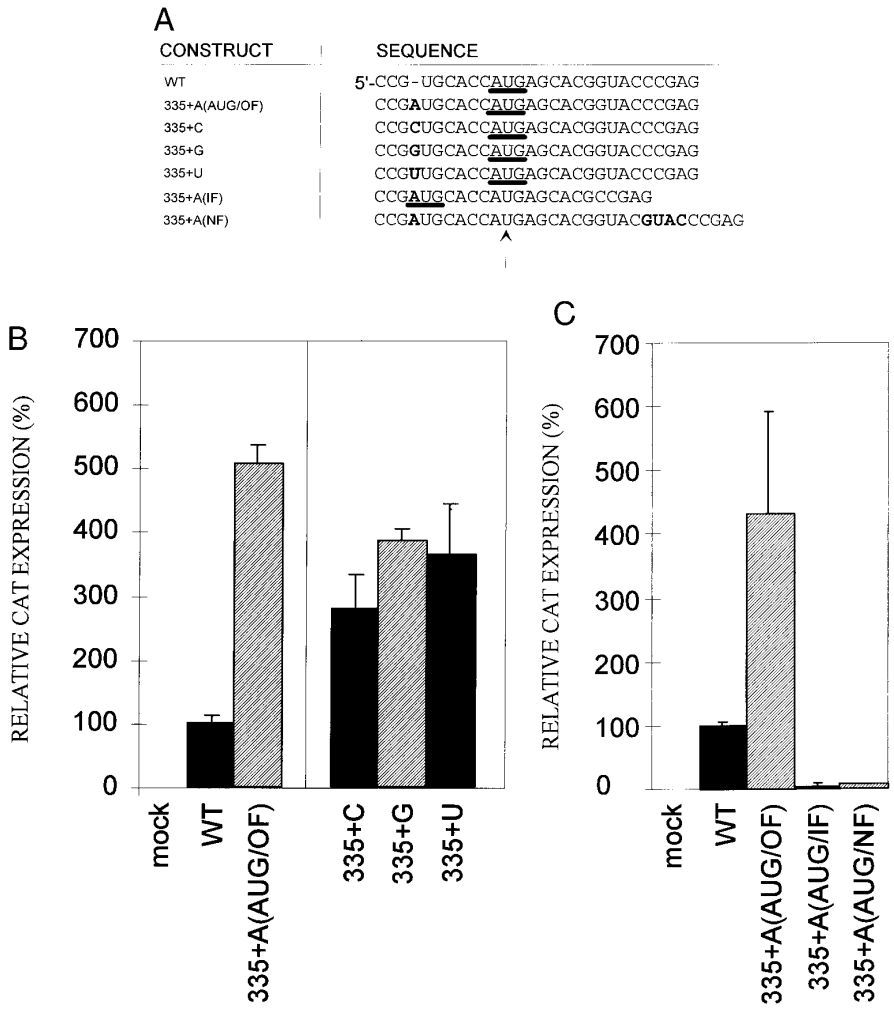

FIG. 4. The effect of mutations just upstream of the AUG codon used for initiating HCV polyprotein synthesis on the HCV 5′ NTR driven expression of CAT protein. (A) Sequence context of the nucleotide insertion mutants at position 335 and the frameshift mutations introduced in p335 + A(IF) and p335 + A(NF). The position of the authentic HCV translation initiation codon is indicated by an arrowhead. The introduced mutations are shown in boldface. AUG codons in frame with CAT gene are underlined. The suffix OF or IF and NF in p335 + A(. . .) indicates that the AUG codons created by this insertion are either out of frame (OF) or in the same reading as the CAT gene (IF). In the NF derivative neither the created or the authentic AUG codon are in the same reading frame as the CAT gene name The GAG triplet at the 3′ end is the first codon of the CAT gene. (B) The relative amount of CAT protein produced in vaccinia vTF7-3 infected Hep2 cells transfected with p335 + A(OF)-CATT, p335 + C-CATT p335 + G-CATT p335 + U-CATT, or pWT-CATT. The expression obtained in pWT-CATT transfected cells was set at 100%. (C) The effect of frameshift mutations introduced in p335 + A(OF)-CATT on the *in vivo* expression of CAT.

(p335+A(OF)-CATT; Fig. 4A; OF, out of frame). Scanning ribosomes are expected to initiate at this additional AUG (AUG^{335}), but as this AUG codon is not in the same reading frame as the polyprotein initiation site (AUG^{orf}), initiation at AUG^{335} would result in a decrease of CAT expression. To our surprise, transfection of this mutant in vTF7-3 infected Hep2 cells resulted in a sixfold enhancement of translation (Fig. 4B; 335+A(OF)).

As AUG^{335} is not in frame with the CAT reporter gene it is very unlikely that recognition of this AUG codon by scanning ribosomes caused the translation enhancement. To obtain direct proof that ribosomes cannot initiate translation at AUG^{335}, plasmid p335+A(IF)-CATT was created. In this mutant AUG^{335} is placed in frame (IF) and AUG^{orf} out of frame with the CAT gene due to a frameshift that was introduced at the border of the HCV capsid sequences and the CAT gene (Fig. 4A). As control for any spurious translation initiation at non-AUG codons p335 + A(NF)-CATT was constructed in which both AUG^{335} and AUG^{orf} are out of frame with the CAT gene (NF: no AUG in frame; Fig. 4A). Transfection of p335+A(IF)-CATT into vTF7-3 infected Hep2 cells resulted in a 10-fold reduction of CAT expression when compared to pWT-CATT. As transfection with p335+A(NF)-CATT gave rise to comparable amounts of CAT protein, this residual CAT expression is most likely due to the initiation of translation at non-AUG codons (Fig. 4B).

To determine whether the increase in translation observed for 335+A(OF)-CATT was due to the creation of an AUG codon or the insertion of any nucleotide at this

position, three additional mutants were made that have a cytidine, a guanosine, or a uridine residue inserted at position 335 (Fig. 4; 335+C, 335+G, 335+U). For these mutants a fourfold higher CAT expression compared to WT-CATT transfected cells was observed (Fig. 4C), suggesting that the enhancing effect is due to the insertion of one nucleotide. Northern blot analysis demonstrated that the introduced mutations did not affect CAT mRNA abundance in the transfected cells (data not shown).

On the basis of these results we conclude that HCV IRES mediated translation does not involve the entry of ribosomes at or close to hairpin C, respectively, followed by ribosomal scanning until AUG^{orf} is encountered. The data we obtained with the AUG^{335} mutants strongly suggest that ribosomes enter the RNA downstream of position 335 and this positions the entry site at or very close to the HCV polyprotein initiation codon.

Engineered AUG codons located downstream of the polyprotein initiation site are not recognized by ribosomes

To determine whether ribosomes are able to recognize an AUG codon located downstream of AUG^{orf}, an AUG codon was engineered just downstream of the *Kpn*I/Asp718 restriction site by inserting the sequence AUGAG. By inserting this pentamer the sequence context of the HCV-H AUG codon is reproduced (ACC-AUG-AGC). Mutant pΔAUG^{orf}-CATT was constructed, in which the polyprotein initiation site is inactivated by replacing the uridine residue at position 343 by a cytidine residue (Fig. 5A, ΔAUG^{orf}). AUG codons were placed in frame with the CAT reporter gene at 10, 14, and 18 nucleotides downstream of the position of the mutated AUG^{orf} in pΔAUG^{orf}-CATT (pΔAUG^{orf}+10AUG-CATT, pΔAUG^{orf}+14AUG-CATT, and pΔAUG^{orf}+18AUG-CATT; Fig. 5). CAT expression of the mutants and WT-CATT was analyzed in vTF7-3 infected and DNA transfected Hep2 cells. The results depicted in Fig. 5B clearly demonstrate that inactivation of AUG^{orf} reduces CAT expression fivefold. This indicates that initiation does occur at non-AUG codons. The insertion of downstream AUG codons did not result in an increase of translation compared to ΔAUG^{orf}-CATT. In fact translation is even further decreased in the presence of these AUG codons (Fig. 5B). Northern blot analysis revealed that comparable amounts of RNA could be detected (data not shown).

The data obtained with these constructs clearly show that ribosomes are not able to initiate translation at AUG codons located as close as 10 nucleotides downstream of the position of AUG^{orf}.

The engineered AUG codons are functional in the absence of the HCV IRES

It is unlikely that all of the engineered AUG codons are not recognized by ribosomes due to a poor sequence context as pΔAUG^{orf}+14AUG-CATT and pΔAUG^{orf}+18AUG-CATT have nucleotides directly surrounding the AUG codon that are identical to the HCV polyprotein initiation site. However, to exclude this possibility we tested translation of several AUG mutants in the absence of a functional HCV IRES. By removing the 5′ 278 nucleotides of the HCV 5′ NTR the IRES domain is inactivated and translation of RNAs obtained from these plasmids therefore results from 5′ end-dependent scanning. CAT expression of the constructs tested in this context is similar to the ΔIRES-WT-CATT level (Fig. 6) except for ΔIRES-ΔAUG^{orf} (Fig. 6), which has no

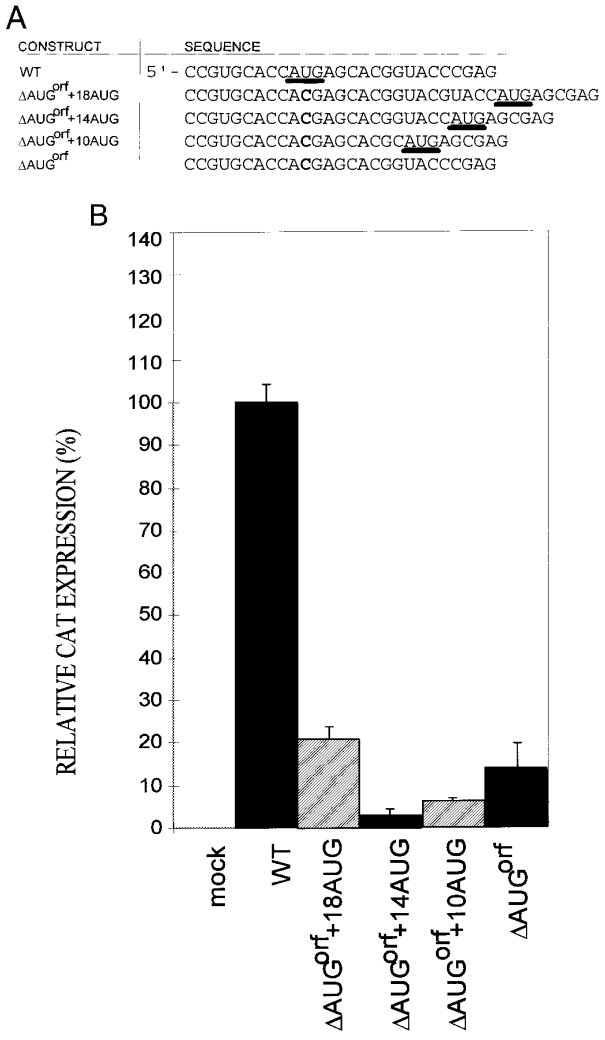

FIG. 5. Analysis of HCV 5′ NTR driven CAT synthesis initiating from AUG codons inserted downstream of the position HCV translation initiation codon. (A) Relevant part of the nucleotide sequence of the analyzed mutants. The U → C mutation introduced to inactivate the authentic HCV AUG codon is indicated in boldface. The AUG codons in frame with the CAT gene are underlined. The number in the name of the mutant indicates the position of the introduced AUG relative to the adenosine residue of the authentic HCV translation initiation codon. Plasmid ΔAUG^{orf} does not contain an in-frame AUG codon upstream of the CAT gene. (B) Relative amount of CAT protein produced in vaccinia vTF7-3 infected Hep2 cells transfected with the plasmids described in A. CAT expression obtained in pWT-CATT transfected cells was set at 100%.

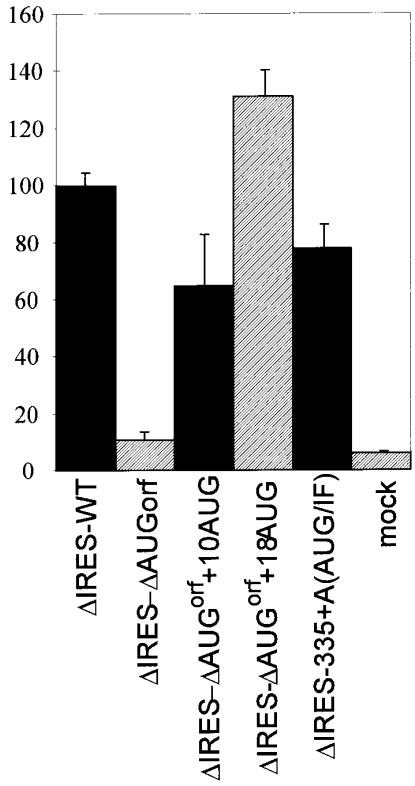

FIG. 6. HCV IRES independent CAT expression starting at AUG codons inserted either up- or downstream of the authentic HCV translation initiation site. CAT expression obtained in pΔIRES-WT-CATT transfected cells was set at 100%.

AUG codons in frame with the CAT gene. This clearly shows that the engineered AUG codons can be recognized by scanning ribosomes.

DISCUSSION

To evaluate whether ribosomes enter the HCV 5′ NTR at or near the conserved hairpin C and whether scanning for the HCV polyprotein initiation site does occur we created mutations in the AUG codons that are present within the HCV-H 5′ NTR and introduced AUG codons at several positions surrounding the polyprotein initiation site.

The results obtained with these mutants show that only the integrity of AUG^{85} and AUG^{96} is important for IRES activity as point mutations that change these AUG codons are detrimental. AUG^{13} is located in hairpin A and for this hairpin it has been demonstrated that it decreases IRES directed translation in both HCV (Rijnbrand *et al.,* 1995) and CSFV (Rijnbrand *et al.,* 1996). Differences in translation efficiency have been observed between HCV genotypes (Tsukyama-Kohara *et al.,* 1992). Some of the nucleotide differences between these genotypes are located within the 5′ NTR but whether these changes correlate with translation efficiency is not known. Two of the nucleotide changes result in the absence of AUG^{13} and AUG^{32} in genotype 1b. Our data show that these two AUG codons do not contribute to the difference in translation efficiency between the two genotypes (Wang *et al.,* 1993; Tsukiyama-Kohara *et al.,* 1992). AUG^{96} is not present in genotype 3 but does have a significant influence on translation efficiency, suggesting that genotype 3 might have decreased translational characteristics. AUG^{215} is located in a region in the HCV 5′ NTR that resembles the conserved Yn-AUG domain in picornaviruses. Inactivation of this AUG codon has only a marginal effect on translation as was also observed by Wang *et al.* (1994). This pyrimidine-rich tract that is present in HCV is, however, absent in pestiviruses, suggesting that the observed similarity with picornaviruses is coincidental.

Mutations in either AUG^{85} or AUG^{96}, both located in or near hairpin C, proved to be inhibitory for HCV 5′ NTR driven translation. A possible explanation is that the 40S ribosomal subunit enters the HCV RNA at or near hairpin C and that both AUG codons are required for the entry process. As a consequence of this model ribosomes have to be transferred to the translation initiation codon that is located circa 250 nucleotides downstream of hairpin C. The observation that a mutant RNA lacking hairpins A, B, and C is translated with an efficiency similar to that of an RNA containing the full-length IRES is consistent with a model in which ribosomes enter the HCV IRES at or near hairpin C and scan the RNA until AUG^{orf} is encountered. However, two additional results make this model very unlikely. First, mutating AUG^{215} to a non-AUG codon did not result in an increase of CAT production and second, an engineered out-of-frame AUG codon 7 nucleotides upstream of AUG^{orf} did not give rise to a decrease but a sixfold increase in CAT expression. The engineered AUG codon at position 335 was also not recognized by ribosomes when this AUG codon was placed in frame with the CAT gene. Strikingly in the absence of a functional HCV-IRES, AUG^{335} was recognized by ribosomes as efficient as AUG^{orf} in WT-CAT RNA. It could be argued that the differences in translation efficiency in the mutants are due to a difference in RNA structure. We consider this unlikely as the mutations were chosen outside of the RNA structures and the modifications between the different mutants are very small. The simplest explanation is that the actual ribosome entry site of the HCV IRES is located downstream of position 335.

As indicated, AUG^{85} is very well conserved in HCV and pestiviruses. However, the amino acid sequence of the encoded protein is not conserved and differs in length between 4 and 19 amino acids (a.a.) in HCV, 23 a.a. in classical swine fever virus (CSVF-Brescia) and 11 a.a. in bovine viral diarrhea virus (BVDV-Osloss). A role in IRES mediated translation of this potential polypeptide is therefore unlikely. AUG^{85} is part of a stretch of 37 nucleotides that has a high degree of similarity with pestiviruses (Han *et al.,* 1991) and might therefore be part of a domain involved in RNA–protein interactions that are essential for IRES functioning. AUG^{96} is located at the 3′ end of

this conserved domain. Although AUG^{96} is not present in pestiviruses and HCV isolates belonging to genotype 3, it can still be argued that the reduced CAT expression observed for $p\Delta AUG^{96}$-CATT results from a lower affinity of the mutated RNA for proteins that are essential for IRES mediated translation.

For picornaviruses two types of IRES elements have been described: type I is present in rhino- and enteroviruses and type II in aphtho-, cardio-, and hepatoviruses. Typical for the type II IRES is that the initiator AUG codon is part of the IRES (Glass *et al.,* 1993; Jang and Wimmer, 1990; Kühn *et al.,* 1990), while in type I IRES elements the polyprotein initiation codon is located 40 to 160 nucleotides downstream of the IRES domain. For poliovirus (type I IRES) and FMDV (type II IRES) it has been shown that scanning plays a role in the recognition of the polyprotein initiation sites (for a recent review see Jackson and Kaminski, 1995). The activity of the picornavirus type I IRES element is highly dependent on the presence of an AUG codon that in contrast to the type II IRES element is not used as the polyprotein initiation site. A common feature of both types of IRES elements is that a specific spacing between this AUG codon and an upstream pyrimidine tract is important for IRES activity. Our results with mutants having engineered AUG codons either up- or downstream of the HCV polyprotein initiation codon strongly suggest that the position of AUG^{orf} is restricted to a certain domain that is close to or at the 3′ end of the IRES. This is further supported by the observation that insertion of 32 nucleotides or a deletion of 9 nucleotides just upstream of HCV-AUG^{orf} strongly reduces translation (Wang *et al.,* 1993, 1995). Recently, Wang *et al.* (1995) have described the presence of an RNA pseudoknot at the 3′ end of the IRES that is an essential element of the HCV IRES. This pseudoknot is also predicted for pestiviruses and we have recently demonstrated that in CSFV the proposed pseudoknot is required for IRES mediated translation (Rijnbrand *et al.,* 1996). It is possible that the pseudoknot or another structure within the HCV and pestiviruses 5′ NTR has a function similar to that of the pyrimidine tract in picornaviruses and that the spacing between this structure and the polyprotein initiation codon is essential for IRES function. The results obtained with the engineered AUG mutants at positions −7, +10, +14, and +18 show that ribosomes enter the HCV RNA in the vicinity of AUG^{orf}. This situation is reminiscent of the type II picornavirus IRES elements, but in contrast to the aphthovirus FMDV for which it has been demonstrated that scanning can occur up to 84 nucleotides downstream of the authentic P20 initiation codon.

Our data strongly suggest that the ribosome binds directly at or very close to the AUG codon that functions as the polyprotein initiation site and if scanning does occur this is limited to a region 6 nt upstream to 9 nt downstream of AUG^{orf}. In pestiviruses an AUG codon is present 7 nucleotides upstream of the polyprotein initiation site. This AUG codon cannot be used as a translation initiation site by ribosomes (Rijnbrand *et al.,* 1996) and an engineered AUG codon located 19 nucleotides downstream of AUG^{orf} could not be recognized by ribosomes to initiate translation. These observations indicate that the inability to initiate translation at up- or downstream located AUG codons is universal for this type of IRES element and that the limited or even absent ribosomal scanning might be a common feature in the mechanism of translation initiation of these related viruses. Yoo *et al.* (1992) and Wang *et al.* (1995) were unable to detect IRES activity in constructs containing circa 30 nucleotides between the HCV 5′ NTR and the initiator AUG codon. This fits with our model because in both cases the AUG codon is at a position where it can no longer be recognized by ribosomes.

Still some controversy exists on the location of the 3′ border of the HCV IRES. Recently, Reynolds *et al.* (1995) claimed that the 3′ border of the HCV IRES is located between nucleotides 13 and 31 of the HCV capsid gene. In several other studies concerning the 3′ border of the HCV IRES no or only 13 additional nucleotides of the HCV core encoding region were incorporated to demonstrate IRES activity (Wang *et al.,* 1993; Rijnbrand *et al.,* 1995; Kettinen *et al.,* 1994). The basis for this discrepancy is unclear. Nevertheless, we determined the influence of additional capsid sequences on AUG recognition and obtained identical results as described for the constructs containing only 8 nucleotides of the HCV capsid gene (data not shown).

A remarkable observation was made when constructs were tested that had no AUG codons in frame with the CAT reporter gene. CAT could still be detected for some constructs, suggesting that non-AUG codons were used to initiate translation, as was also observed by others (Reynolds *et al.,* 1995). This initiation at non-AUG codons was most efficient when the CAT reporter gene was in the same reading frame as the capsid gene. This indicates that preferably initiation at an in-frame non-AUG codon occurred, most likely the ACG codon engineered to mutate AUG^{orf}.

There are two different models to envision how ribosomes select the AUG codon for initiating HCV polyprotein synthesis. In the first model, an 80S ribosomal complex is formed directly at the polyprotein initiation site without any additional scanning. In the second model a 43S ternair complex initially binds to a domain just upstream of the polyprotein initiation site. This can be mediated by cellular proteins like PTB that has been shown to bind to HCV 5′ NTR (Ali and Siddiqui, 1995) and for which it has been demonstrated to play an essential role in IRES mediated translation of the picornavirus EMCV RNA (Kaminski *et al.,* 1995). Next, the ribosomal subunit could scan to the polyprotein initiation site where the 80S ribosome is assembled.

As we are unable to detect translation initiation of the AUG codons engineered just downstream of AUG^{orf} the second model is very unlikely. For the first model the AUG codon is an essential part of the HCV IRES and the finding that recognition of non-AUG codons occurs although at a much lower efficiency is consistent with this. If the 43S ternair complex is positioned very close to the polyprotein initiation site and the actual binding is dependent on the presence of an AUG codon, then in the absence of AUG codons binding of the 43S ternair complex could take place at codons that differ in only one nucleotide from an AUG codon as has also been described for cap dependent translation (Kozak *et al.,* 1989; Peabody *et al.,* 1987).

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